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BIOTECHNOLOGY and BIOENGINEERING

Modeling Bacterial Contamination of Fuel Ethanol Fermentation

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Received 26 September 2008; revision received 5 December 2008; accepted 15 December 2008

Published online 30 December 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22244

ABSTRACT: The emergence of antibiotic-resistant bacteria may limit the effectiveness of antibiotics to treat bacterial contamination in fuel ethanol plants, and therefore, new antibacterial intervention methods and tools to test their application are needed. Using shake-flask cultures of *Saccharomyces cerevisiae* grown on saccharified corn mash and strains of lactic acid bacteria isolated from a dry-grind ethanol facility, a simple model to simulate bacterial contamination and infection was developed. Challenging the model with 10^8 CFU/mL *Lactobacillus fermentum* decreased ethanol yield by 27% and increased residual glucose from 6.2 to 45.5 g/L. The magnitude of the effect was proportional to the initial bacterial load, with 10^5 CFU/mL *L. fermentum* still producing an 8% decrease in ethanol and a 3.2-fold increase in residual glucose. Infection was also dependent on the bacterial species used to challenge the fermentation, as neither *L. delbrueckii* ATCC 4797 nor *L. amylovorus* 0315-7B produced a significant decrease in ethanol when inoculated at a density of 10^8 CFU/mL. In the shake-flask model, treatment with 2 μg/mL virginiamycin mitigated the infection when challenged with a susceptible strain of *L. fermentum* (MIC for virginiamycin $\leq$2 ppm), but treatment was ineffective at treating infection by a resistant strain of *L. fermentum* (MIC = 16 ppm). The model may find application in developing new antibacterial agents and management practices for use in controlling contamination in the fuel ethanol industry.

Biotechnol. Bioeng. 2009;103: 117–122.

Published 2008 Wiley Periodicals, Inc.

KEYWORDS: bacterial contamination; fuel ethanol; lactic acid bacteria; *Lactobacillus fermentum*

Introduction

Bacterial contamination is a continual problem in commercial fermentation cultures, particularly in fuel ethanol fermentations that are not performed under sterile, pure-culture conditions (Connolly, 1997). A variety of Gram-positive and Gram-negative bacteria have been isolated from fuel ethanol fermentations including species of *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, and *Clostridium* (Lushia and Heist, 2005; Skinner and Leathers, 2004). The most common offending microbes, however, are species of *Lactobacillus*, whose fast growth rate and tolerance to alcohol and low pH allow them to effectively compete with the yeast (Makanjuola and Springham, 1984; Skinner and Leathers, 2004). Chronic bacterial contamination poses a constant drain on sugar available for conversion to ethanol and the bacteria scavenge essential micronutrients required for optimal yeast growth and ethanol production. Acute infections occur unpredictably, and bacterial byproducts such as acetic and lactic acids inhibit yeast growth and may result in "stuck" fermentations that require costly shut-downs of facilities for cleaning (Makanjuola et al., 1992; Narendranath et al., 1997). Despite efforts to prevent contamination with extensive cleaning and disinfecting procedures, saccharification tanks, continuous yeast propagation systems, and notoriously resistant biofilms can act as reservoirs of bacteria that continually reintroduce contaminants (Skinner and Leathers, 2004). For this reason, antibiotics are frequently used to prevent and treat contamination (Bayrock et al., 2003; Day et al., 1954; Stroppa et al., 2000).

The most common commercially available products used to control contamination in fuel ethanol facilities are based on the antibiotics virginiamycin and penicillin (Connolly, 1997; Lushia and Heist, 2005). The emergence of drug-resistant strains, however, may limit the effectiveness of these agents. Decreased susceptibility to virginiamycin has

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been observed in *Lactobacillus* isolated from dry-grind ethanol plants that use virginiamycin, and the emergence of isolates with multi-drug resistance to both virginiamycin and penicillin has been reported (Bischoff et al., 2007; Lushia and Heist, 2005). Therefore, new antibacterial agents and new drug management methods will need to be developed to effectively control bacterial infections.

The present work was initiated to develop tools to provide a fundamental understanding of acute and chronic bacterial contamination of commercial fuel ethanol plants. Previously published studies on bacterial contamination have used chemostat bioreactors to model commercial fermentation processes (Bayrock and Ingledew, 2003; Narendranath et al., 1997, 2001). Bioreactors allow control of pH and substrate feeding as well as continuous monitoring of various fermentation parameters, but they are expensive to operate in terms of equipment costs and labor, and therefore are not amenable to high throughput screening. The work presented here describes a simple shake-flask culture model of *Saccharomyces cerevisiae* experimentally contaminated with strains of lactic acid bacteria isolated from a dry-grind ethanol facility to simulate bacterial contamination and infection.

Materials and Methods

Strains and Media

For the growth of *S. cerevisiae*, YP/glucose media contained the following per liter: 10 g yeast extract, 20 g peptone, and 170 g glucose. Difco Lactobacilli MRS Broth base was manufactured by Becton, Dickinson, and Co. (Sparks, MD). *S. cerevisiae* strain NRRL Y-2034 was obtained from the ARS Culture Collection maintained at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, IL) (McGhee et al., 1982). *Lactobacillus delbrueckii* ATCC 4797 was obtained from the American Type Culture Collection (Manassas, VA). It is not an ethanol plant isolate, but was used to compare the response of the ethanol plant strains to a non-adapted strain. *L. fermentum* strains 0315-1, 0315-8, 0315-11, and 0315-25, *L. brevis* strain 84, *L. amylovorus* 0315-7B, and *Weisella confusa* 0216-1 were isolated from fermentors at commercial dry-grind ethanol facilities located within the Midwestern United States. The isolation procedure for planktonic and biofilm isolates was performed as described previously (Skinner and Leathers, 2004; Skinner-Nemec et al., 2007). Sixty-two planktonic isolates and 10 biofilm isolates were picked and streaked for isolation three times prior to testing for identification. The strains were identified by sequencing of 16s rRNA genes. Amplicons were generated by polymerase chain reaction using the following primer set: U1 (CCAGCAGCCGCGGTAATACG) and U2 (ATCGGYTACCTTGTTACGACTTC) (Lu et al., 2000). The resulting product (about 1,000 bp) was purified using a Qiagen (Valencia, CA) PCR purification kit, and one strand was sequenced by standard methods with the U1 primer. The sequences obtained were compared with those in the GenBank database by using the BLASTN program (Altschul et al., 1997) available at the National Center for Biotechnology Information (available at http://www.ncbi.nlm.nih.gov). More than 98% identity to a known species was considered a positive match.

Shake-Flask Fermentation

Stock cultures of *S. cerevisiae* were grown in YP media supplemented with 5% (w/v) glucose at 32°C and 200 rpm. Stock cultures of lactic acid bacteria were grown in static cultures of MRS media at 37°C. Cells were harvested by centrifugation, and inocula prepared by resuspending cells in sterile saline solution to a density of $OD_{600\ nm}$ equivalent to 80 and $OD_{600\ nm}$ equivalent to 8.0 for *S. cerevisiae* and bacteria, respectively. One $OD_{600\ nm}$ of *S. cerevisiae* corresponds to $\sim 6 \times 10^7$ CFU/mL, and one OD_{600nm} of *Lactobacillus* sp. is equivalent to $\sim 1 \times 10^8$ CFU/mL.

Corn mash (~33% solids) was obtained from a commercial dry-grind ethanol facility and stored at −20°C. The facility uses ~50% backset in the mash, and the pH was adjusted within the range of 5.0–5.2 with sulfuric acid. The mash was collected after the cooking process with a hydro heater temperature of ~107°C. The liquefaction dextrose equivalency range was 8–12, and the initial concentrations of glucose, acetic acid, and lactic acid were 7.1, 0.36, and 0.46 g/L, respectively. Direct plating of mash samples on MRS agar did not detect transient bacteria in the mash ($<10^2$ CFU/mL).

Mash (40 mL) was dispensed in a 50 mL Erlenmeyer flask, and supplemented with ammonium sulfate (0.12%, w/v) and glucoamylase (20 μL of Optidex L-400; Genencor International Inc., Rochester, NY). The culture was inoculated with 0.5 mL of *S. cerevisiae* inoculum, and when indicated, challenged with 0.5 mL of bacterial inoculum. Enzyme, yeast, and when indicated, bacteria and virginiamycin were added sequentially at time 0. Flasks were capped with a rubber stopper and a 20-gauge needle inserted between the stopper and flask to allow for venting of CO_2. Cultures were incubated at 32°C with gentle shaking (100 rpm) for 72 h.

Analytical Methods

Bacterial density was enumerated by colony counting on MRS agar media containing cycloheximide (10 μg/mL). Concentrations of ethanol, glucose, lactic acid, and acetic acid were determined by high-performance liquid chromatography using a 300 mm Aminex HPX 87H column (Bio-Rad Laboratories, Inc., Hercules, CA) on a HP 1100 Series HPLC system equipped with a refractive index detector (Agilent Technologies, Santa Clara, CA). Samples (10 μL) were injected onto a heated column (65°C) and eluted at 0.6 mL/min using 5 mM H_2SO_4 as mobile phase. Concentrations are reported as mean values (±standard deviation) of at least triplicate cultures. Statistical comparisons of

challenged and control cultures were performed using Student's t-test ($P < 0.05$).

Results and Discussion

Fermentation

In a contamination model, there are a number of variables to be examined, including the feedstock, bacterial strain, bacterial load, and any potential antibacterial treatment. Although microprocessor-controlled bioreactors are generally good for simulating the industrial fermentation process at the laboratory scale, their high cost in terms of equipment, media consumption, and labor limit the practical number of combinations of these variables. Therefore, we sought to develop a simple bacterial contamination model using shake-flask fermentations challenged with bacterial strains isolated as contaminants of fuel ethanol facilities.

Initial fermentations used 17% (w/v) glucose in a yeast extract/peptone media challenged with a mixture of three *L. fermentum* strains. These strains were originally isolated as contaminants from a dry-grind ethanol plant experiencing an acute infection episode. Final ethanol concentrations in the challenged cultures were only 2% less than the unchallenged (Table I). Although lactic and acetic acid concentrations were significantly higher ($P < 0.05$) in challenged cultures, the final concentration of 2.8 g/L lactic acid was less than the "alarm value" of 8 g/L widely accepted in the industry (Abbott and Ingledew, 2004; Narendranath et al., 2001). The final pH values were correspondingly lower in challenged (pH 4.3) versus controls (pH 4.8). There was also no difference in residual glucose, with the challenged culture essentially using all available glucose, presumably providing greater biomass at the expense of ethanol production. It is possible that the low-viscosity YP/glucose media had a higher capacity for dissolved oxygen and maintained an aerobic environment, which reduced the response to the contaminating bacteria. Nevertheless, the use of glucose in YP media may represent the state of a chronic bacterial infection, but does not effectively simulate the state of acute infections.

Insoluble matter and protein in corn mash increases its buffering capacity. Using exogenously added acids, Abbott and Ingledew (2004) concluded that the buffering capacity of whole corn mash mitigates the inhibitory effects of lactic and acetic acids on *S. cerevisiae*. Nevertheless, in our model, the deleterious effect caused by experimentally infected contaminants was much greater with saccharified corn mash as feedstock than with glucose. Ethanol yields were reduced 27% and residual glucose increased from 6.2 to 45.3 g/L at 48 h in control and challenged cultures, respectively (Table I). Lactic and acetic acid concentrations in challenged cultures increased to 5.2 and 3.1 g/L, respectively, and the final pH dropped from 4.5 in control cultures to 4.0 in experimentally infected ones. This suggests that with the strains used here, the mash's buffering capacity was not sufficient to encumber the effects of the acids. A synergistic inhibitory effect on yeast-catalyzed fermentations has previously been observed when both lactic and acetic acids are present (Narendranath et al., 2001), which may explain the response difference observed in the present study between the glucose and corn mash feedstocks. It is also possible that lactic acid bacteria outcompete yeast by effectively scavenging the limited micronutrients available in corn mash. It should be noted that final bacterial load in the corn mash fermentations were 10-fold higher than in the glucose fermentations ($3.8 \pm 1.5 \times 10^9$ CFU/mL vs. $1.5 \pm 0.5 \times 10^8$ CFU/mL). Thus, the corn mash provided a more hospitable environment for the lactic acid bacteria relative to *S. cerevisiae*, allowing the contaminants to effectively compete with the yeast.

Based on a previous survey of bacterial contaminants of corn-based fuel ethanol facilities in the United States that found the bacterial load in dry-grind plants could reach 10^8 CFU/mL (Skinner and Leathers, 2004), we initially challenged the shake-flask cultures with 10^8 CFU/mL *L. fermentum*. In yeast-catalyzed fermentations of wheat mash in bioreactors, Narendranath et al. (1997) reported that challenging with up to 10^9 CFU/mL lactobacilli (corresponding to a 1,000:1 ratio of lactobacilli to yeast) resulted in as much as a 7.6% decrease in final ethanol concentration. In the present study, we challenged the corn mash fermentations with varying concentrations of *L. fermentum* 0315-1 ranging from 10^4 to 10^8 CFU/mL, which corresponds to lactobacilli:yeast ratios of 1:1,000 to 10:1. Final ethanol decreased and residual glucose increased as the inoculation of *L. fermentum* increased (Fig. 1). The final ethanol concentration was 17% lower with 10^8 CFU/mL *L. fermentum* inoculum, but even the 10^5 CFU/mL

Table I. Challenge of *S. cerevisiae* fermentations with *L. fermentum*.[a]

Feedstock	Challenge	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
YP/glucose	−	75.7 ± 0.353	0.396 ± 0.324	0.129 ± 0.005	0.459 ± 0.037
	+	74.1 ± 0.477	0.501 ± 0.324	2.79 ± 0.135	1.15 ± 0.032
Corn mash	−	112 ± 2.00	6.2 ± 1.3	1.0 ± 0.1	0.8 ± 0.1
	+	81.8 ± 2.40	45.5 ± 1.18	5.2 ± 0.1	3.1 ± 0.1

Data are reported for 48 h fermentations (YP/glucose) or 72 h fermentations (corn mash). Data are mean values ± standard deviation from three replicate cultures.

[a]Initial density of *S. cerevisiae* was approximately 10^7 CFU/mL. Cultures were challenged with the following *L. fermentum* strains each at a density of 10^7 CFU/mL: 0315-1, 0315-8, and 0315-11.

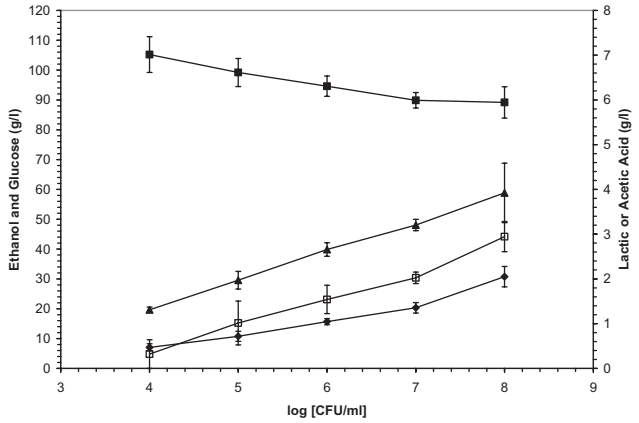

Figure 1. Dose–response on ethanol fermentation by varying inoculum of *L. fermentum* 0315-1. Cultures of *S. cerevisiae* grown on corn mash feedstock were challenged with the indicated inoculum of *L. fermentum* 0315-1. The following products were measured after 72 h incubation: ethanol (■), residual glucose (□), lactic acid (▲), acetic acid (◆). Data are reported as mean values ± standard deviation of triplicate cultures. Final concentrations for the control culture (no bacterial challenge) were as follows: ethanol = 108 ± 3 g/L, glucose = 4.8 ± 3.6 g/L, lactic acid = 0.63 ± 0.01 g/L, and acetic acid = 0.31 ± 0.04 g/L.

challenge decreased ethanol 8%. There were no significant differences ($P > 0.05$) in final ethanol and residual glucose between the 10^4 CFU/mL challenge and unchallenged controls. Narendranath and Power (2004) concluded increasing the yeast inoculation rate reduces the growth of contaminating lactic acid bacteria, with a rate of 3×10^7 cells/mL able to outcompete a 10-fold increase in bacterial inoculation. Thus, the effect of lactobacilli contamination observed in the present study is more pronounced than that reported previously (Narendranath et al., 1997; Narendranath and Power, 2004). While previous studies used strains of *L. plantarum* and *L. paracasei* to challenge corn mash fermentations in bioreactors, the isolates used here may be more potent antagonists of yeast-catalyzed fermentation.

Strain Specificity

We tested six strains of lactic acid bacteria in the shake-flask model (Table II). *L. fermentum* 0315-1, *L. fermentum* 0315-25, and *L. brevis* 84 produced the most deleterious effects on the yeast-catalyzed fermentation. These isolates were obtained from a fermentor sample during an acute infection at the ethanol facility, where strains of *L. fermentum* and *L. brevis* accounted for 85% (53 of 62) and 13% (8 of 62) of the planktonic isolates examined, respectively. *L. amylovorus* 0315-7B and *L. delbrueckii* ATCC 4797 did not significantly change the final ethanol and residual glucose concentrations, although lactic acid concentrations did increase. Another lactic acid bacterium, *W. confusa* 0216-2, produced a small but significant decrease in ethanol and increase in residual glucose. Although increases in lactic acid concentrations were roughly equal for all six strains tested, it is noteworthy that *L. amylovorus*, *L. delbrueckii,* and *W. confusa* produced the smallest increases in acetic acid production. *L. amylovorus* and *L. delbrueckii* are homofermentative bacteria, while *L. fermentum*, *L. brevis*, and *W. confusa* are heterofermentative (Hammes and Hertel, 2006). The change in acetic acid levels reported in Table II is consistent with the fermentation type of the bacterial species. Thus, acetic acid may be a determining factor in decreasing ethanol yield. Narendranath et al. (1997) and Narendranath and Power (2004) have suggested that lactic acid, acetic acid, and ethanol act synergistically to inhibit yeast fermentation. It is possible that the negligible effects caused by these lactobacilli are related to lower acetic acid production, but strain-specific fitness issues may also play a role. As noted above, *L. fermentum* accounted for the majority of strains isolated from the fermentor, but only one strain of *L. amylovorous* was isolated, and it was obtained during the biofilm sampling. At the end of the fermentation, *L. fermentum* 0315-1 was present at 10^9 CFU/mL, but *L. amylovorus* was present at only 9×10^2 CFU/mL and neither *L. delbrueckii* nor *W. confusa* were detectable in the beer ($<10^2$ CFU/mL). Narendranath et al. (1997) observed that the death rate of lactobacilli increased in the later stages of yeast-catalyzed fermentations, suggesting that ethanol

Table II. Challenge of *S. cerevisiae* with lactic acid bacteria.[a]

Strain	*N*	Δ Ethanol (g/L)	Δ Glucose (g/L)	Δ Lactic (g/L)	Δ Acetic (g/L)
L. fermentum 0315-1	7	−15.8	31.4	2.88	1.52
L. fermentum 0315-25	3	−16.1	28.7	2.52	1.27
L. brevis 84	3	−29.6	54.1	4.90	2.17
L. amylovorus 0315-7B	3	−5.00*	ND*	3.94	−0.025*
L. delbrueckii ATCC 4797	3	−0.458*	ND*	2.30	0.0889
W. confusa 0216-2	10	−3.3	7.87	1.96	0.515

Data are reported as the change in mean values of the indicated parameter between challenged cultures and unchallenged controls. *N* indicates the number of replicate cultures. "ND" indicates that residual glucose concentrations were not detectable. An asterisk denotes that the mean values between challenged and unchallenged controls were not significantly different ($P > 0.05$).

[a]Cultures used corn mash as feedstock and were inoculated with approximately 10^7 CFU/mL *S. cerevisiae*. Cultures were challenged with the indicated strains at a density of 10^7 CFU/mL.

Table III. Experimentally infected cultures treated with virginiamycin.[a]

Challenge	MIC[b] (μg/mL)	Treatment[c]	Ethanol (g/L)	Glucose (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
None	NA	−	107 ± 3.96	1.7 ± 0.73	0.780 ± 0.070	0.394 ± 0.114
		+	109 ± 2.72	2.0 ± 1.1	0.740 ± 0.030	0.433 ± 0.035
L. fermentum 0315-1	16	−	88.7 ± 3.27	35.0 ± 7.59	3.71 ± 0.291	2.02 ± 0.438
		+	87.4 ± 5.24	33.2 ± 5.58	3.55 ± 0.349	1.88 ± 0.290
L. fermentum 0315-25	≤2	−	85.2 ± 2.56	37.5 ± 2.45	3.93 ± 0.333	2.12 ± 0.310
		+	109 ± 2.12	1.2 ± 0.47	1.22 ± 0.243	0.499 ± 0.013

NA, not applicable; ND, not detected.

[a]Cultures of *S. cerevisiae* grown on corn mash feedstock were challenged with the indicated strain at an initial density of 10^8 CFU/mL. Reported concentrations are values at 72 h incubation ± standard deviation from three replicate cultures.

[b]Minimum inhibitory concentration for virginiamycin.

[c]Cultures marked as "−" were not treated; cultures marked as "+" were treated with 2 μg/mL virginiamycin.

and lactic acid act synergistically to kill the bacteria, especially at lower pH. In contrast here, *L. fermentum* increased in density 10-fold over the initial inoculation rate, suggesting that it is a particularly aggressive antagonist of *S. cerevisiae*-catalyzed fermentation.

Treatment With Virginiamycin

Virginiamycin is one of the most common commercially available antibiotics used to treat and prevent bacterial contamination in the fuel ethanol industry (Hynes et al., 1997). The recommended dosing range of this agent in fuel ethanol fermentations is generally 0.25–2.0 ppm. To validate the shake-flask model for testing antibacterial treatments, we used a virginiamycin-susceptible strain (*L. fermentum* 0315-25, MIC ≤2 μg/mL) and a virginiamycin-resistant strain (*L. fermentum* 0315-1, MIC = 16 μg/mL) to experimentally infect the model, and treated the fermentations with 2 μg/mL virginiamycin. Virginiamycin had no detectable effect on fermentation by *S. cerevisiae* in unchallenged control fermentations (Table III). Both *L. fermentum* strains reduced ethanol yield and increased residual glucose to a similar extent in untreated fermentations, but virginiamycin treatment only mitigated the infection of strain *L. fermentum* 0315-25. Virginiamycin treatment did not significantly affect ethanol yield, residual glucose, or lactic and acetic acid concentrations when challenging with the virginiamycin-resistant strain *L. fermentum* 0315-1.

Conclusions

In the present study, we have demonstrated the ability to produce infections of corn mash in shake-flask cultures using selected strains of lactic acid bacteria. The magnitudes of ethanol loss and residual glucose were dependent on the infecting strain and on the initial bacterial load. Treatment with virginiamycin mitigated the effects of infection by a susceptible strain, suggesting that the strains and fermentation parameters described here may prove suitable for testing novel antibacterial control methods. The simplicity of the model allows for miniaturization into multi-well plate formats, and given the dynamic range of the residual glucose response and the availability of commercial devices for rapid glucose monitoring, it may be developed for high-throughput screening processes to test new antibacterial agents. Application of this model to the study of contamination will help develop a fundamental understanding of acute and chronic bacterial contamination events.

Expert technical assistance was provided by Melinda S. Nunnally, Eric Hoecker, and Imran Khan. This work was funded in part by the National Science Foundation (Award # 0538739 to R.E.W.) and by the State of Illinois Matching Fund.

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